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Low Density Lipoprotein Receptor as a Candidate Receptor for Hepatitis C Virus

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Hepatitis C virus (HCV) binds to different human cell lines in vitro. However, the efficiency of adsorption is very low due mainly to a relatively small fraction of the virus being able to bind to these cells. Free low density lipoprotein (LDL > 200 μg/ml) is able to block the attachment of HCV to human fibroblasts in vitro completely. COS-7 cells being primarily not able to bind HCV were transfected with a vector containing the entire coding sequence of the human LDL-receptor (LDLR). HCV was now bound to these cells. We propose that HCV and LDL are competitive for the cellular LDLR and that LDL in sera of patients may regulate the binding of HCV to this target. ***J. Med. Virol. 57:223–229, 1999.***

KEY WORDS: HCV; LDL; LDL-receptor; β-lipoprotein; cell culture; virus–cell interaction

INTRODUCTION

Hepatitis C virus (HCV) has been shown to be the major causative agent responsible for most cases of parenteral non-A, non-B hepatitis [Choo et al., 1989; Kuo et al., 1989]. A large number of HCV-infected patients develop chronic hepatitis, which eventually leads to cirrhosis and may progress to hepatocellular carcinoma [Saito et al., 1990].

The HCV genome is a positive-sense, single-stranded RNA molecule of approximately 9,400 bases containing an open reading frame encoding a 3,010–3,011 amino acid polyprotein [Houghton et al., 1991]. The polyprotein displays the greatest homology with the flavivirus and pestivirus members of the *Flaviviridae* family; however, the sequence divergence and putative polyprotein structure would suggest that HCV represents a novel virus type that might constitute a new member of this family [Miller and Purcell, 1990; Choo et al., 1991].

The pathogenesis of HCV infection is not well understood because there have been few attempts to study HCV replication in vitro. Evidence of in vitro replication has been shown in different human cell lines: MOLT-4 cells, HPB-Ma cells, Daudi and Huh-7 cells [Shimizu et al., 1993, 1996], and H9 cells [Nissen et al., 1994]. In other studies, replication of HCV was observed in fetal hepatocytes, peripheral blood mononuclear cells, human fibroblasts VH3, and in a human bone-marrow-derived B-cell line [Bertolini et al., 1993; Iovacci et al., 1993, 1997; Cribier et al., 1995; Zibert et al., 1995; Seipp et al., 1997]. However, there is no satisfactory in vitro model of HCV infection, because significant release of HCV particles is not detectable in any of these cell cultures.

Heterogeneities with respect to buoyant density and sedimentation of HCV RNA-carrying material (HCVrcm) have been reported in different groups of patients, and have been partly attributed to the binding of viral particles to β-lipoproteins (low density lipoprotein [LDL], very low density lipoprotein [VLDL]) and immunoglobulins (IgG/IgM) [Thomssen et al., 1992, 1993; Hijikata et al., 1993; Prince, 1994; Agnello, 1995; Prince et al., 1996]. We discussed previously the role of lipoproteins in persistence and virus–cell interactions of HCV [Thomssen et al.,1993; Monazahian et al., 1995]. In this study, we report that the addition of purified LDL (>200 μg/ml) inhibits the binding of HCV to human fibroblasts completely. Transfection of African green monkey kidney cell COS-7 with a vector containing the entire coding sequence of the human LDL-receptor (LDLR) [Yamamoto et al., 1984; Hofer et al., 1994] enables HCV to bind to these cells. These experiments suggest a possible role of the LDLR as a receptor for HCV.

MATERIALS AND METHODS

Patient Sera

The sera of patients with chronic active hepatitis C used in our experiments were derived from the diagnostic laboratory of this department. They are positive for HCV-RNA and for anti-HCV antibodies.

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Cell Culture

Normal neonatal human dermal fibroblasts (HSF), LDLR-deficient human fibroblasts (FH), and COS-7 cells (SV40-transformed Africa green monkey kidney cells; Gluzman, 1981) were grown at 37°C/5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l of glucose supplemented with 10% fetal calf serum (FCS), 584 mg/l L-glutamine, and antibiotics (penicillin, 100 U/ml, streptomycin, 100 μg/ml). Human T-cell leukemia line Molt-4 cells were grown in suspension in RPMI 1640 supplemented with 10% FCS and antibiotics.

Virus Binding Assay

Adherent growing cells were seeded into a 3-cm diameter culture dish and were incubated for 48 hr in DMEM supplemented with 10% lipoprotein-deficient serum (LPDM) to reach about 70–80% confluence. Cells were then washed with phosphate-buffered saline (PBS) and incubated for 3 hr at 37°C in 1 ml culture medium containing 100 μl HCV-positive serum. Following incubation the supernatant was removed. Cells were washed five times with 1 ml PBS and once with 200 μl PBS (last washing step). Cells were then detached from the culture dish using trypsin (0.5 g/l)/ethylenediamine tetraacetic acid (EDTA) (0.2 g/l) solution and a rubber cell scraper, collected in 200 μl PBS, and diluted in two-fold steps in 200 μl PBS.

Molt-4 cells (10^7) were incubated with 500 μl HCV-positive inoculum, in 2 ml RPMI 1650 culture medium for 2 hr at 37°C/5% CO_2. Following incubation the cells were pelleted (5 min at 1,000 × *g*), washed, and diluted as described above. RNA was isolated from the last washing step and the cell dilutions and HCV-RNA was detected by reverse transcription-polymerase chain reaction (RT-PCR). The proportion between internalized or membrane-bound virus and the virus titer in the supernatant is described by the adsorption quotient (AQ %).

Detection of HCV by RT-PCR

RNA was isolated from 200 μl of inocula or cell dilutions using the RNA-Clean system (AGS, Heidelberg, Germany). One-fifth (2 μl) of the RNA was used for RT. The cDNA synthesis was performed at 37°C for 90 min in a total volume of 20 μl containing the reaction buffer (10 mM Hepes pH 6.9, 0.2 mM EDTA pH 8, 50 mM Tris-Cl pH 7.5, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM dNTP), 20 U RNase inhibitor (RNA-guard, Pharmacia), 15 U Superscript II-RT (GIBCO/BRL, Bethesda, MD), and 50 ng Primer NTR-74 (5′CCCAACACTACTCGGCTAGCAGTC3′). The reaction was stopped by heat inactivation of the RT for 3 min at 100°C. One-fourth (5 μl) of the cDNA was amplified by 30 cycles of PCR using 0.5 U of Taq-Polymerase (Boehringer Mannheim) and 30 ng of each primer, NTR-74 and NTR-318 (5′ACTCCACCATGAATCACTCCCC3′) in a total volume of 50 μl reaction mix (10 mM Tris-Cl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM dNTP). Each cycle consisted of 60 sec at 94°C, 60 sec at 56°C, and 30 sec at 72°C followed by an extension for 7 min at 72°C after the last cycle. A second PCR was carried out with 1 μl of the first-round product under the same experimental conditions but using 125 ng of each internal primer, NTR-279 (5′TTCACGCAGAAAGCGTCTAGCC3′) and NTR-113 (5′CAAATCTCCAGGCATTGAGCGGG3′). One-tenth of the second-round product was analyzed by agarose gel electrophoresis.

HCV-RNA titer was determined by a semi-quantitative method [Thomssen et al., 1992, 1993] by titrating cDNA in four-fold steps. For each step one PCR was carried out. Virus titers are expressed as dilution factors (i.e., a titer of 256 refers to a positive PCR result of a 1:256 diluted cDNA). This semi-quantitative method also allows an estimation of the number of copies of HCV-RNA per milliliter plasma/serum.

Transfection of COS-7 Cells

COS-7 cells were transfected with vectors pSVL-LDLr(+) or pSVL-LDLr(−), respectively, containing the entire coding sequence of the human LDLR in sense or antisense orientation [Hofer et al., 1994]; 10^6 cells were seeded into a 3-cm diameter culture dish and chamber slides then grown overnight to reach a confluence of about 50%. Transfection was carried out using transfection reagent DOTAP (Boehringer Mannheim, Germany) following the manufacturer's recommendations with 3 μg DNA. After 4 hr, the transfection medium was replaced by culture medium supplemented with 10% FCS and sterols (12 μg/ml Cholesterol and 2 μg/ml 25-hydroxycholesterol) to suppress expression of endogenous LDLR. After 24 hr, the medium was removed. The control cells in the chamber slides were tested for the expression of LDLR by labeling with 10 μg/ml DiO-LDL (Biogenesis) for 5 hr, fixation in 3% formaldehyde/PBS, and fluorescence microscopy. The cells grown in the culture dishes were incubated for 2 hr at 37°C with 1 ml culture medium containing 100 μl HCV-positive serum, and then treated as described above.

Coprecipitation of HCVrcm With Anti-LDL-Antibody

Serum (100 μl) was mixed with 100 μl anti-LDL-antibody (Sigma, St. Louis, MO) or PBS incubated at 4°C overnight. Precipitates were sedimented (10 min at 5,000 × *g*) and HCV-RNA was determined in the starting material, in the supernatant and sometimes additionally in the sediment by PCR [Thomssen et al., 1992]. HCV-RNA titer was determined by titrating cDNA in four-fold steps. One PCR was carried out for each step.

RESULTS

Binding of HCVrcm to LDL

A group of HCV-positive sera of differing HCV-RNA amounts was precipitated with anti-LDL-antibody at +4°C (Fig. 1). The sera contained titer between 4 to

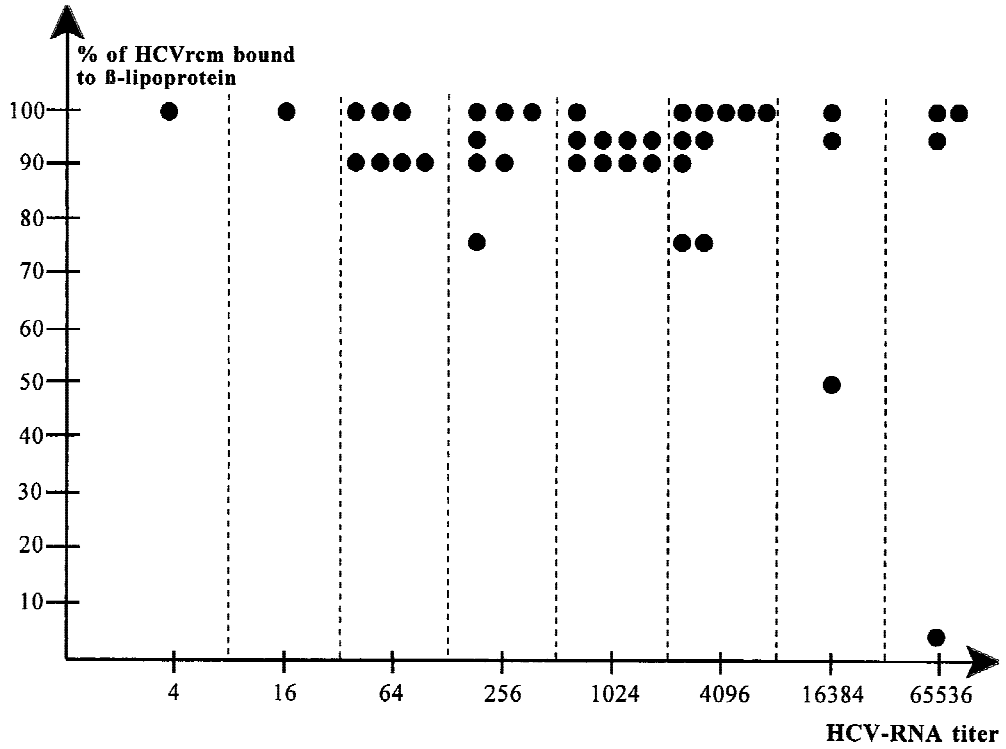

Fig. 1. Association of hepatitis C virus RNA-carrying material (HCVrcm) with β-lipoprotein (LDL) in 42 sera (●) of different HCV-RNA titer. The coprecipitation of HCVrcm with anti-LDL-antibodies was carried out as described in Materials and Methods. The HCV-RNA titer in the supernatant was determined by limiting dilution reverse transcription-polymerase chain reaction (RT-PCR). In the case that HCVrcm was completely bound to LDL (100%), no HCV-RNA was detectable in the supernatant after coprecipitation with anti-LDL-antibodies. In most cases HCVrcm could be coprecipitated with anti-LDL-antibodies at 4°C nearly completely, but in some sera only a weak association of HCVrcm with LDL was detectable.

65,536 corresponding to 10^2–10^6 HCV-genome-equivalents per milliliter. Most of the HCVrcm could be precipitated (>90%). In this group, only one serum showed no interaction with LDL. The binding efficiency did not depend on the HCV-RNA-titer.

Adsorption of HCVrcm to Molt-4 Cells and Human Fibroblasts

According to the results of other researchers, we examined the binding of HCV to Molt-4 cells and human fibroblasts in vitro [Shimizu et al., 1993; Zibert et al., 1995]. The efficiency of adsorption was very low. The adsorption quotient (AQ) of the 25 surveyed sera varied from 0 to 4.7% (Table I). The titer of HCV-RNA in the used sera had no influence on the amount of the adsorbed virus. We also found no correlation between the percentage of bound virus and the quantity of HCVrcm in the sera that could be precipitated with anti-LDL-antibody (data not shown).

Inhibition of HCVrcm Binding to Human Fibroblasts by LDL

Assuming that HCVrcm might be adsorbed to the LDLR [Thomssen et al., 1992], we tried to inhibit the HCV-cell interactions by LDL. Because human fibroblasts bind HCVrcm and are well-recognized tools in LDLR research [Brown and Goldstein, 1986], we used them for further experiments. The existence of LDLR in the membranes of the normal human dermal fibroblasts (HSF) used in our experiments was confirmed by immunofluorescence with DiO-LDL. The inhibiting ac-

TABLE I. Binding Capacity of HCVrcm to Molt-4 Cells and HSF*

Molt-4			HSF		
Sera	Titer[a]	AQ[b] (%)	Sera	Titer[a]	AQ[b] (%)
1	4,096	0.1	a	256	0.8
2	256	2.3	b	1,024	0.4
3	256	1.9	c	4,096	0.1
4	640	1.9	d	1,024	0.3
5	16,384	0.1	e	4,096	0.1
6	4,096	1.9	f	4,096	0.1
7	256	4.7	g	256	0.4
8	4,096	0.5	h	4,096	0
9	4,096	0.5	i	1,024	3.0
10	1,024	0.5	j	256	0
11	256	0.3	k	4,096	0
12	256	0	l	16,000	1.3
			m	4,096	3.0

*HCVrcm, hepatitis C virus RNA-carrying material; HSF, human dermal fibroblasts.
[a]Detection of HCV-RNA by limiting dilution reverse transcription-polymerase chain reactions.
[b]AQ, adsorption quotient.
Adsorption is defined by detection of HCV-RNA in cells while supernatant of the last washing step is HCV-RNA negative. In most cases only a minority of virus (<1%) was bound. The AQ describes the proportion between bound virus and the virus titer in the supernatant.

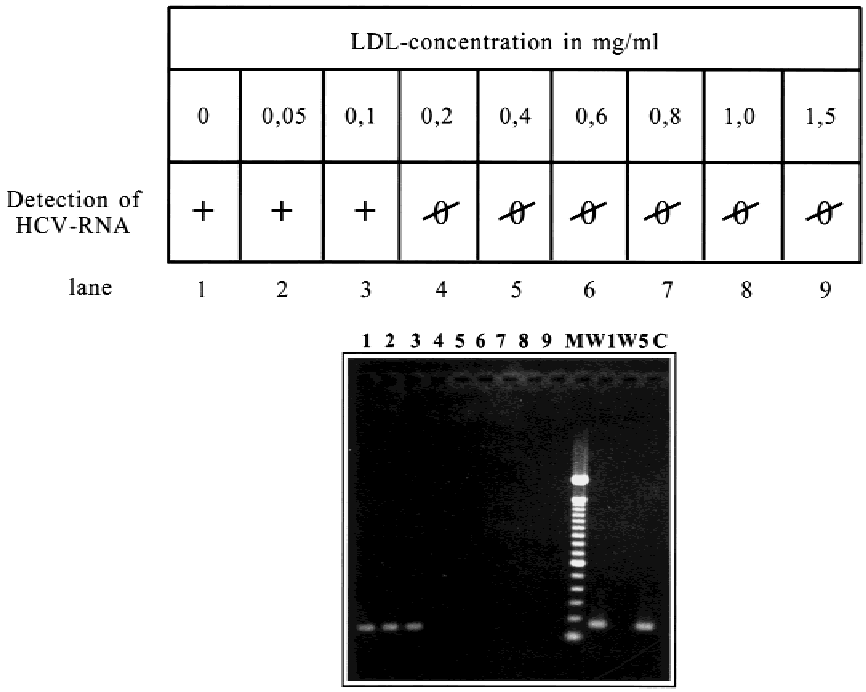

Fig. 2. Inhibition of hepatitis C virus (HCV) binding to human fibroblasts (HSF) by purified low density lipoprotein (LDL). Cells were incubated 30 min with different concentrations of LDL before the binding assay was carried out. HCV-RNA in HSF was detected by reverse transcription-polymerase chain reaction (RT-PCR). The adsorption of HCV RNA-carrying material (HCVrcm) to HSF can be inhibited completely by adding >200μg/ml purified LDL. Agarose gel: M, molecular weight marker; C, positive control; W1, first wash step; W5, 5th wash step.

tion of purified human LDL (Sigma) on the binding of HCVrcm to HSF was examined. Cells were incubated for 30 min with different concentrations of LDL before the binding assay was carried out. The adsorption of HCVrcm to HSF could be inhibited completely by adding > 200 μg/ml purified LDL (Fig. 2).

In addition, no interaction was found of HCVrcm to LDLR-deficient human fibroblasts (FH) from a patient with familial hypercholesterolemia (Table II).

TABLE II. Incubation of Human Fibroblasts (HSF) and LDLR-Deficient Human Fibroblasts (FH) with 5 HCV-RNA Positive Sera

	Cell lines	
Sera	HSF	FH
E.	+	–
S.	+	–
D.	–	–
B.	+	–
G.	–	–

The binding assay was carried out as described in Materials and Methods. Although hepatitis C virus RNA was detectable in three cases even in 1:4 diluted HSF (+), no HCVrcm was bound to FH (–).

Comparison of HCVrcm Binding to pSVL-LDLr(+)- and pSVL-LDLr(–)-Transfected COS-7 Cells

Based on the previous results, it was postulated that the LDLR may play a decisive role in the reception of the virus. To obtain more evidence, we carried out a virus binding assay with COS-7 cells transfected with pSVL-LDLr(+) and pSVL-LDLr(–), respectively. Under conditions that suppress synthesis of endogenous LDLR, in 7 of 12 experiments with different sera, HCVrcm adsorbed to pSVL-LDLr(+)-transfected cells but not to pSVL-LDLr(–)-transfected cells (Fig. 3). Virus binding was demonstrable with three sera both to the pSVL-LDLr(+)- and pSVL-LDLr(–)-transfected COS-7 cells. No virus–cell interaction could be demonstrated in two further cases (Table III).

DISCUSSION

Only sparse data are available concerning the cellular uptake of the HCV, the replication cycle, the mechanism of persistence, and even the morphology of the viral particle [Shimizu et al., 1992; Carloni et al., 1993; Prince et al., 1996]. The observation that HCVrcm is associated with LDL [Thomssen et al., 1992], VLDL [Agnello et al., 1995; Prince et al., 1996], and immunoglobulins [Hijikata et al., 1993; Shimizu et al., 1993; Thomssen et al., 1993] led to the assumption that these associations may favor binding of the virus to the target cells and support the persistence of HCV infections.

LDL is internalized by LDLR-mediated endocytosis [Brown and Goldstein, 1986]. We presumed that HCV likewise interacts with the LDLR. The binding of HCVrcm to human fibroblasts (HSF) could be inhibited by purified LDL (>200 μg/ml, Fig. 2). The results of the binding assay with pSVL-LDLr(+)- and -LDLr(–)-transfected COS-7 cells further strengthen our hypothesis (Fig. 3). In 7 of 12 sera examined an HCV binding

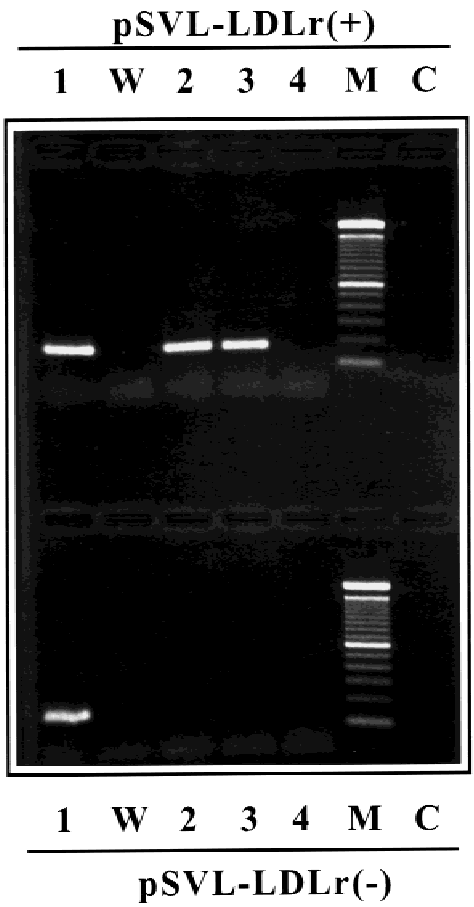

Fig. 3. Comparison of hepatitis C virus (HCV) binding to pSVL-LDLr(+) and pSVL-LDLr(−) transfected COS-7 cells. Cells were incubated with HCV-RNA-positive sera for 2 hr and washed five times with phosphate-buffered saline (PBS). Then they were harvested and diluted and RNA was extracted from cells and the last washing step. The results of HCV reverse transcription-polymerase chain reaction (RT-PCR) (NCR-region) are shown. Agarose gel: lane 1, positive serum cell supernatant ; W, last wash step; lane 2, cells diluted 1:4; lane 3, cells diluted 1:8; lane 4, cells diluted 1:16; M, molecular weight marker; C, negative control.

was found only to COS-7 cells that express the human LDLR on their surface (Table III). Correspondingly, fibroblasts derived from a patient with familial hypercholesterolemia lacking LDLR did not bind HCV (Table II). However, a comparison between HSF and LDLR-deficient fibroblasts (FH) is restricted due to a reduced growing and metabolic activity of the FH that may influence the binding capacity.

The LDLR is the main representative of a family of cell-surface receptors (LDLR gene family) that share characteristic sequences, for example a ligand-binding domain [Brown et al., 1997]. Members of the LDLR gene family function as receptors for minor-group common cold virus [Hofer et al., 1994] and for subgroup A Rous sarcoma virus [Bates et al., 1993]. This points to the possibility that the LDLR (or members of the gene family) might also function as a receptor for HCV. However, although the ability of the LDLR to bind HCVrcm was demonstrated, it is uncertain that virus uptake is mediated by this receptor. The cell culture

TABLE III. Incubation of COS-7 Cells Transfected With Vectors pSVL-LDLr(+) or pSVL-LDLr(−) With 12 HCV-RNA-Positive Sera

Sera	pSVL-LDLr(+)	pSVL-LDLr(−)
N.	++	++
Re.	++	++
Re.	+	+
Bu.	+	−
Bu.	+	−
Ro.	++	−
Bo.	++	−
Bj.	+	−
D.	+	−
O.	+	−
J.	−	−
D.	−	−

HCV, hepatitis C virus.
The binding assay was carried out as described in Materials and Methods under conditions that suppress synthesis of endogenous low density lipoprotein receptor (LDLR). Cells were washed, detached and diluted in two-fold steps. Each dilution step was tested for HCV-RNA by reverse transcription-polymerase chain reaction (RT-PCR). (++) points to a positive PCR result up to a 1:8 dilution and (+) to a positive result up to a 1:2 dilution. No HCV-RNA was detectable even in undiluted cells as indicated by (−).

system used is not suitable for infection studies and detection of replicative HCV intermediates.

In 3 of 12 sera binding of HCVrcm to pSVL-LDLr(−)-transfected COS-7-cells was observed (Table III), even under conditions that suppress an expression of endogenous LDLR [Davis et al., 1987]. At present, it must be considered that there are other receptors for HCVrcm belonging to the LDLR gene family or others, which may also mediate the binding and uptake of HCVrcm.

HCVrcm normally has a low binding-efficiency to animal and human cell lines in vitro. The quantity of membrane-bound or internalized virus was found to be low even if sera with high HCV-RNA titers were used for the binding-assay (Table I). It is unlikely that this low efficiency is due to a restriction at the cellular level, because the amount of cells in each test well was clearly higher than the calculated number of membrane-bound or internalized HCVrcm. Besides, prolongation of incubation times did not lead to an increased adsorption quotient (data not shown).

It has been impossible so far to identify and characterize the HCV virion. The observed heterogeneity of HCVrcm in regard to density or size results from an association of the putative virion with β-lipoproteins [Thomssen et al., 1992; Prince, 1994] or human immunoglobulins [Hijikata et al., 1993]. But some fractions that tested positive for HCV-RNA may also represent populations of naked capsids [Kaito et al., 1994; Kanto et al., 1994] or defective particles. It remains unclear, which proportion of the HCVrcm constitutes the infectious particles. Therefore, the observed low adsorption quotient may express the small population of infectious HCVrcm in a given serum.

It is also necessary to consider whether HCVrcm is bound directly to LDLRs or whether lipoproteins bound to the virus may function as a ligand. In most HCV-RNA-positive sera, HCVrcm could be coprecipitated

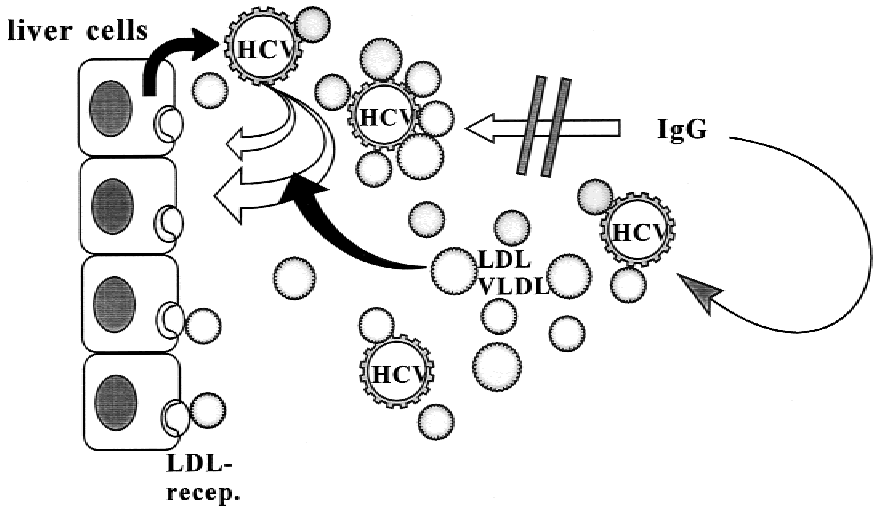

Fig. 4. Model of the binding of serum proteins to hepatitis C virus (HCV) and possible infection of the liver cells (see Discussion).

with β-lipoproteins (LDL, VLDL) at +4°C (Fig. 1). Favoring the ligand hypothesis, one would expect a clearly increased adsorption quotient. But we found recently that the binding of HCVrcm to β-lipoproteins depends strongly on the temperature of reaction. At 37°C, binding of the majority of the virus in a given serum cannot be observed, but in most of these sera there exists a small fraction of HCVrcm that is bound to LDL even at 37°C. We rarely found HCVrcm-positive sera, where most of the virus was bound to β-lipoproteins at 37°C (data not shown). The adsorption studies were carried out at 37°C, at which temperature most of the HCVrcm used in our adsorption experiments was not bound to β-lipoproteins. However, we have shown that there are small fractions of HCVrcm in each serum that can be precipitated by anti-LDL-antibodies despite the high temperature. This question cannot be answered as yet, but it seems possible that the virus not only can be bound directly to the cells but also by means of a lipoprotein ligand already bound to the virus.

Combining the previous results relating the interaction between lipoproteins, LDLR, and HCV a model is suggested for HCV persistence (Fig. 4). Virions are released by liver cells and may infect other liver cells via LDLRs. Free β-lipoproteins in a human serum being in the order of 0.6–1.6 mg/ml may regulate the rate of the infection of liver cells by competing with the virus. This competition may contribute to the mechanism of persistency of the infection. Whether there are additional effects of the binding of lipoproteins to HCVrcm, as, for instance, delaying the induction of neutralizing antibodies or inhibiting their action and thus contributing to persistence, is an open question. Heterogeneities of the virus particles with regard to differences in the structure of the outer membranes, and the absence of surface proteins to which neutralizing antibody is directed or in the size of the virus particles ("interfering particles") [Prince et al., 1996], may influence the persistence of infection.

Research in the field of cellular receptors of HCV is limited by the low binding efficiency of this virus and the absence of a cell culture system that generates high amounts of infective virus particles [Shimizu et al., 1997]. The results represented in this study and previously by Thomssen et al. [1992, 1993] and Prince et al. [1994] once more illustrate the great heterogeneity of HCV in different serum samples. Further analysis of HCV-RNA positive sera and the characterization of different isolates of the virus are necessary to gain a deeper insight into the nature of HCV infection.

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